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Transcriptional Profiling of *Vibrio cholerae* Recovered Directly from Patient Specimens during Early and Late Stages of Human Infection

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Understanding gene expression by bacteria during the actual course of human infection may provide important insights into microbial pathogenesis. In this study, we evaluated the transcriptional profile of *Vibrio cholerae*, the causative agent of cholera, in clinical specimens from cholera patients. We collected samples of human stool and vomitus that were positive by dark-field microscopy for abundant vibrios and used a microarray to compare gene expression in organisms recovered directly from specimens collected during the early and late stages of human infection. Our results reveal that *V. cholerae* gene expression within the human host environment differs from patterns defined in in vitro models of pathogenesis. *tcpA*, the major subunit of the essential *V. cholerae* colonization factor, was significantly more highly expressed in early than in late stages of infection; however, the genes encoding cholera toxin were not highly expressed in either phase of human infection. Furthermore, expression of the virulence regulators *toxRS* and *tcpPH* was uncoupled. Interestingly, the pattern of gene expression indicates that the human upper intestine may be a uniquely suitable environment for the transfer of genetic elements that are important in the evolution of pathogenic strains of *V. cholerae*. These findings provide a more detailed assessment of the transcriptome of *V. cholerae* in the human host than previous studies of organisms in stool alone and have implications for cholera control and the design of improved vaccines.

Bacterial behavior in the host is influenced by nutrient availability and by environmental substrates that change as infection progresses and tissue breakdown and inflammation occur. These factors influence bacterial growth rate and population dynamics and affect the production of virulence determinants. To date, study of bacterial physiology during the actual course of human infection has been technically difficult. However, the development of highly sensitive microarray-based techniques for evaluating global microbial gene expression has made such an approach feasible. In this study, we compared the gene expression profiles of *Vibrio cholerae* bacteria recovered directly from patient specimens during early and late stages of human infection, using transcriptional profiling by microarray. Our goal was to define virulence factors expressed in the human host and to identify differences with existing models of cholera pathogenesis based on in vitro studies.

V. cholerae, the etiologic agent of cholera, has been extensively studied using in vitro systems. This work indicates that the coordinate expression of a network of pathogenicity genes enables the organism to colonize the small intestine and produce cholera toxin (CTX), which leads to secretory diarrhea (14). In addition to CTX, a second major virulence factor of *V. cholerae* is the toxin-coregulated pilus (TCP), a type IV pilus that is required for intestinal colonization (12, 23). TCP also serves as the receptor for the entry of CTXϕ, the filamentous bacteriophage that encodes cholera toxin (24). In vitro, two transmembrane transcription complexes, ToxRS and TcpPH, have been shown to sense environmental conditions and act through a common downstream regulator, ToxT, to coordinate the simultaneous expression of the genes encoding TCP and CTX (4, 5, 9, 23).

Interestingly, analyses of *V. cholerae* in recently shed human stool specimens have not identified high-level expression of genes encoding CTX or TCP or of genes involved in virulence regulation (3, 19). Rather, compared to in vitro-grown organisms, *V. cholerae* in stool specimens appears to be in a physiologic state of preparation for dissemination into the environment. These findings suggest that transcriptional profiling of the organism in stool specimens may not identify virulence genes essential in the early phases of colonization and pathogenesis in the human. Furthermore, interpretation of the transcriptional profile of *V. cholerae* recovered from stool has been complicated by the lack of a biologically relevant comparator state.

Here we directly compare the transcriptional profile of *V.*

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cholerae in the early phase of infection of the human upper intestine, represented by organisms in vomitus, with that of *V. cholerae* in stool, representing the late phase of human infection. Our results characterize the dynamic physiologic state of *V. cholerae* during the course of human infection and identify key differences from patterns of virulence gene expression identified in vitro.

MATERIALS AND METHODS

Collection of clinical samples. At least 50 ml of stool or vomitus was collected immediately upon passage from patients presenting to the International Centre for Diarrhoeal Disease Research in Bangladesh (ICDDR,B) with *V. cholerae* O1 or O139 infection, prior to the receipt of antibiotics. As previously noted (3, 19), such specimens contain high numbers of the infecting serogroup of *V. cholerae* and are largely free of other organisms. Specimens that were positive by dark-field microscopy for the darting movement of vibrios were plated onto taurocholate-tellurite-gelatin agar for overnight culture and placed directly into Trizol (Life Technologies) for subsequent extraction of total RNA. Two specimens of vomitus and five specimens of stool that were positive by culture for *V. cholerae* O1 or O139 were included in this analysis. Quantitative culture was performed on samples of both stool and vomitus and yielded at least 10^8 CFU/milliliter. Human patients' approval was obtained from the Massachusetts General Hospital and the ICDDR,B.

RNA and genomic DNA extraction. Total RNA was isolated from the clinical samples using Trizol (Life Technologies). RNA samples were treated with DNase to remove contaminating DNA on an RNeasy column (QIAGEN). Quantities of RNA were determined by spectrophotometry, and visualization on a 1% agarose gel was used to verify the integrity of the RNA. Genomic DNA from the sequenced *V. cholerae* O1 El Tor strain N16961 (10) was prepared using the Easy-DNA kit (Invitrogen) according to the manufacturer's instructions.

Microarrays and hybridization. The *V. cholerae* microarray consists of 3,890 full-length PCR products representing the annotated open reading frames from the initial release of the *V. cholerae* N16961 genome (10). The construction of the array, fluorescent cDNA and genomic DNA labeling, hybridization, and data collection were carried out as previously described (3, 6). Each labeling and hybridization was performed in duplicate. Genomic DNA was used as a universal internal control for the quality of the microarray and also allowed for the comparison of results across multiple experiments (22). Genes with insufficient genomic DNA hybridization to the microarray were excluded from the analysis.

Statistical analysis. Data were normalized using locally weighted regression to obtain the relative abundance of each transcript as an intensity ratio with respect to that of genomic DNA (26). High correlation coefficients were observed between technical replicates (Pearson's correlation coefficient [r] > 0.80) and between results for separate clinical specimens of vomitus ($r > 0.77$) and of stool ($r > 0.80$). Hence, the results from the two clinical vomitus specimens and the five clinical stool specimens were pooled, and Welch's t test was used to assess the statistical significance of differences in median *V. cholerae* gene expression between the two phases of human infection. Adjustment for multiple comparisons was made using the false discovery rate control ($P < 0.05$) (2). Fold changes for the relative expression of a given gene between the two types of clinical specimens were calculated by dividing the normalized median intensity ratios with respect to genomic DNA. The full data sets are available as supplementary material via the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/GSE 2775).

Regional clusters of genes were identified by an iterative assessment of all genes evaluated with the microarray; clusters were defined as regional groups in which >70% of genes showed similar expression patterns. The statistical significance of the clustering was calculated according to the hypergeometric distribution.

Quantitative RT-PCR. Quantitative reverse transcription-PCR (RT-PCR) was performed to verify the microarray results for *tcpA* on clinical samples with sufficient remaining material (one clinical vomitus sample and three clinical stool samples). RNA isolated as described above was reverse transcribed using the Reverse Transcription System (Promega). Primers and probes for *tcpA* and *V. cholerae* 16S RNA were designed using Primer Express (Applied Biosystems). Quantitative RT-PCRs were done using the TaqMan system (Applied Biosystems) and an Opticon 2 continuous fluorescence detector (MJ Research). Real-time PCRs were performed in a final volume of 25 μl containing 1× TaqMan Universal Master Mix (Applied Biosystems), 900 nM forward and reverse Taqman primers, and 250 nM Taqman probe. Primers and probes were purchased from Applied Biosystems, and reactions were performed in MicroAmp Optical 96-well plates (Applied Biosystems). Validation experiments were performed for all TaqMan probe and primer sets, and these showed a linear relationship between the cycle threshold (C_T) and the logarithm of the template amount (genomic DNA), as expected. To control for genomic DNA contamination, reactions without reverse transcriptase were performed. Relative expression levels in the different samples were calculated by using the comparative C_T method with 16S RNA as the internal reference for normalization.

RESULTS

Of the 3,882 individual genes evaluated, 42 (1%) were significantly differentially expressed between the two phases of human infection (Table 1). Most of the differentially expressed genes were more highly expressed in early than in late human infection, and many of these genes are involved in DNA replication, energy production, and protein synthesis. These results indicate that early human infection is a period of active replication and metabolic activity for *V. cholerae*.

A number of virulence factors were significantly more highly expressed in the earliest stage of cholera infection (Table 1). The gene with the single most significant difference in expression ($P = 7 \times 10^{-7}$) was *tcpA*, which was >6-fold more highly expressed in early than in late human infection. By quantitative RT-PCR, *tcpA* transcript abundance was >150-fold higher in vomitus than in stool. Although previous studies have demonstrated that TCP is an essential colonization factor of *V. cholerae* (12, 23), this is the first direct evidence of its expression during early human infection.

Two putative hemolysins were also among the virulence factors that were differentially expressed during early human infection (Table 1). *V. cholerae* hemolysins are structurally similar to pore-forming toxins of other bacteria and may contribute to the enterotoxic activity of the organism (13, 25). Notably, 11 hypothetical proteins were identified in our analysis; further study of the role of these proteins in cholera pathogenesis, as well as that of the two putative hemolysins, is warranted.

As with many other pathogenic bacteria, the major virulence genes of *V. cholerae* are clustered in several chromosomal regions; these pathogenicity islands appear to have been acquired in horizontal gene transfer events that have been important in the evolution of pathogenic strains (7, 20). Because of this, we looked for clusters of contiguous genes that were similarly regulated in early or late *V. cholerae* infection. We performed an iterative assessment of all genes evaluated by our microarray; significant clusters were identified as regional groups in which >70% of genes showed similar expression patterns.

Our analysis identified four highly significant clusters of genes ($P < 10^{-7}$), all of which were upregulated during early infection (Fig. 1). One of these clusters, VC2568 to VC2597, comprises ribosomal proteins and likely reflects the particularly active replicative state of *V. cholerae* during early human infection. The second regional cluster of genes is located on the *V. cholerae* small chromosome from VCA0560 to VCA0570 ($P = 4.9 \times 10^{-10}$). This cluster includes a number of hypothetical proteins and a transcriptional regulator, raising the possibility that these genes may represent an operon expressed in response to an environmental signal in human infection. The most significant cluster of genes spans the *V.*

TABLE 1. Individual Vibrio cholerae genes with statistically significant differential expression between early and late human infection[a]

TIGR designation	Protein characterization	Fold change (vomit/stool)	*P*
More highly expressed in early human infection			
Biosynthesis of cofactors: VCA0558	Gamma-glutamyltranspeptidase, putative	3.94	1.1×10^{-6}
Cellular processes (pathogenesis)			
VC0578	Hemolysin, putative	1.62	1.2×10^{-4}
VC0828	Toxin coregulated pilin TcpA	6.61	7.6×10^{-7}
VC0959	Hemolysin, putative	1.80	6.6×10^{-5}
Cellular processes (chemotaxis and motility): VC2601	Sodium-type flagellar protein MotX	1.82	9.2×10^{-5}
DNA metabolism			
VC0394	Excinuclease ABC, subunit A	2.07	4.6×10^{-4}
VC0543	RecA protein	3.94	2.0×10^{-4}
VC1845	Holliday junction DNA helicase RuvB	2.92	9.7×10^{-6}
VC1846	Holliday junction DNA helicase RuvA	4.73	5.0×10^{-4}
VCA0198	Site-specific DNA methyltransferase, putative	2.60	1.1×10^{-5}
Energy metabolism			
VC2413	Pyruvate dehydrogenase, E2 component, dihydrolipoamide	3.24	3.5×10^{-4}
VC2447	Enolase	3.05	2.8×10^{-4}
VCA0897	DevB protein	2.04	1.1×10^{-5}
Hypothetical proteins			
VC1152	Hypothetical protein	1.82	4.9×10^{-5}
VC2365	Hypothetical protein	1.33	3.2×10^{-4}
Hypothetical proteins (conserved)			
VC0480	Conserved hypothetical protein	1.91	1.2×10^{-4}
VC0641	Conserved hypothetical protein	4.90	4.0×10^{-4}
VC0762	Conserved hypothetical protein	1.95	3.4×10^{-4}
VC1317	Conserved hypothetical protein	2.88	3.3×10^{-5}
VC1723	Conserved hypothetical protein	1.84	1.4×10^{-4}
VC2479	Conserved hypothetical protein	1.84	2.1×10^{-4}
VC2706	Conserved hypothetical protein	1.74	2.2×10^{-4}
VC2720	Conserved hypothetical protein	1.60	1.8×10^{-4}
VCA0769	Conserved hypothetical protein	2.26	9.8×10^{-6}
Protein synthesis			
VC2594	Ribosomal protein L23	2.45	1.1×10^{-4}
VC2595	Ribosomal protein L4	3.31	2.0×10^{-4}
Purines, pyrimidines, nucleosides, and nucleotides			
VC0675	Thymidylate synthase	1.84	4.2×10^{-4}
VCA0197	GMP reductase	1.64	8.5×10^{-5}
Regulatory function			
VC2415	Pyruvate dehydrogenase complex repressor	2.72	2.6×10^{-4}
VC2464	Sigma-E factor regulatory protein RseC	2.04	2.9×10^{-5}
Transcription: VC0006	RNase P protein component	1.80	3.3×10^{-4}
Unknown function			
VC1579	Enterobactin synthetase component F-related protein	3.24	1.6×10^{-6}
VC2627	DamX-related protein	1.74	5.8×10^{-5}
VCA0534	DedA family protein, authentic frameshift	1.64	1.2×10^{-4}
VCA0560	GGDEF family protein	2.75	3.8×10^{-7}
More highly expressed in late human infection			
Hypothetical proteins			
VC0666	Hypothetical protein	0.72	4.6×10^{-5}
VC0807	Hypothetical protein	0.54	5.0×10^{-4}
VCA0143	Hypothetical protein	0.62	2.3×10^{-4}
VCA0746	Hypothetical protein	0.68	1.1×10^{-4}
Hypothetical protein (conserved): VC0688	Conserved hypothetical protein	0.70	1.8×10^{-4}
Protein fate: VC2675	Protease HsIVU, subunit HsIV	0.40	2.2×10^{-4}
Protein synthesis: VC1047	Fatty oxidation complex, alpha subunit	0.50	4.0×10^{-4}

[a] After adjustment for multiple comparisons with the false discovery rate control.

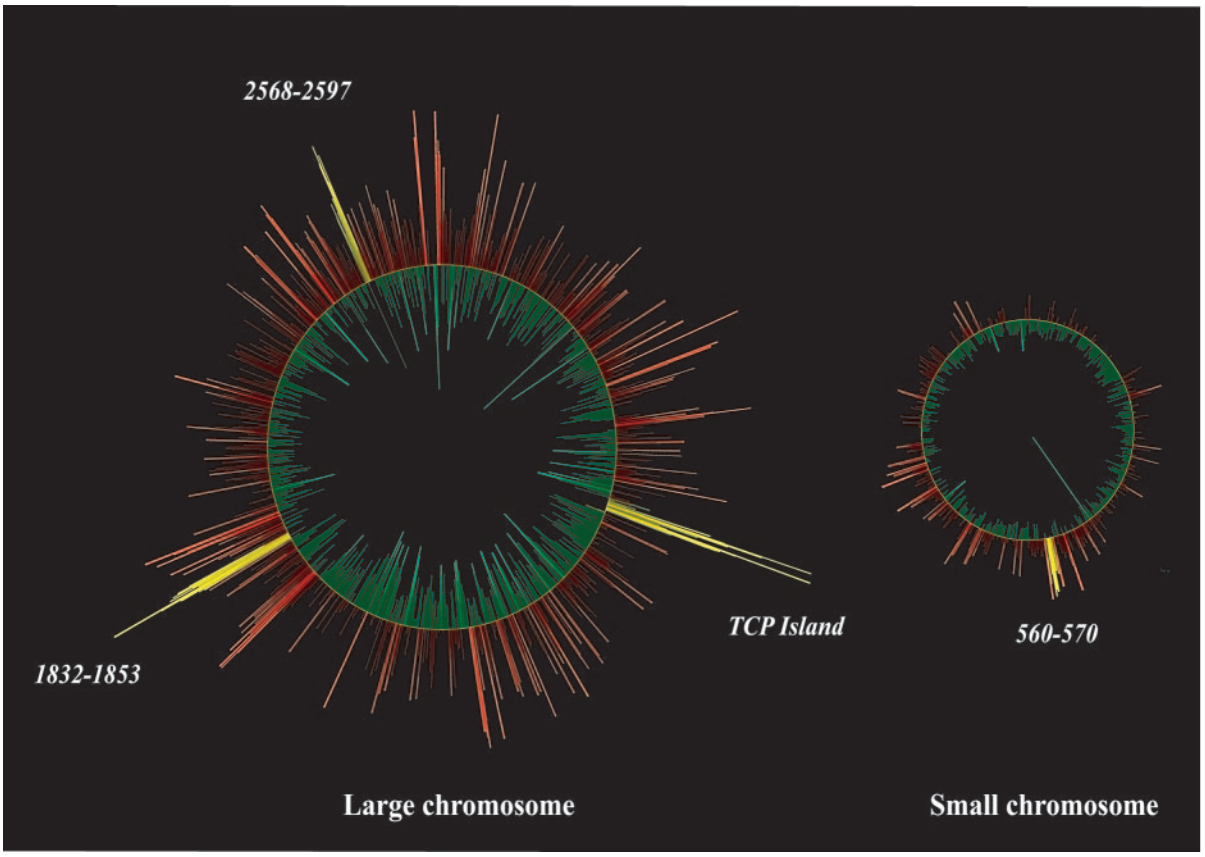

FIG. 1. Regional clusters of genes on the *V. cholerae* large and small chromosomes that display similar patterns of expression during human infection ($P < 10^{-7}$). All genes evaluated with the microarray are shown. For each gene, the $\log_{10}$-fold change in expression in early infection (vomit) compared with late infection (stool) is represented graphically. Genes that are more highly expressed in early human infection are represented in red, and genes that are more highly expressed in late human infection are represented in green. Significant clusters of genes are represented in yellow.

cholerae large chromosome from VC1832 to VC1853 ($P = 1.5 \times 10^{-17}$). Many of these genes are transcribed in the same direction, suggesting that they may be under common control. Included within this region are the contiguous genes *tolQRA*, which together encode a membrane complex that is required for CTXϕ entry into the microbe (11). Two genes contained in this region, *ruvA* and *ruvB*, encode proteins that are essential for homologous recombination (21). *recA*, although not part of this cluster, was also significantly more highly expressed in early than in late human infection (fold change, 3.94 [$P = 2.0 \times 10^{-4}$]). With high levels of expression of *tcpA*, *tolQRA*, and genes involved in homologous recombination, the human upper intestine may therefore be a particularly well suited environment for horizontal gene transfer events that are important in the evolution of pathogenic *V. cholerae* strains.

The final cluster identified in our analysis was the group of genes encoding TCP ($P = 1.35 \times 10^{-9}$) (Fig. 2). Included in this cluster is *tcpA*, which was among the 42 individual genes identified as significantly differentially expressed between the two phases of human infection (Table 1). TCP is part of a previously described 40-kb pathogenicity island that may have been acquired in a horizontal gene transfer event (15, 16). In our microarray studies, 29 of the 31 genes on the TCP pathogenicity island were upregulated in early compared with late infection, including the cytoplasmic transcriptional factor *toxT* (fold change, 12.12 [$P = 0.01$]), although the upregulation of genes other than *tcpA* did not reach individual statistical significance with our small sample size. This suggests that the ToxT-regulated expression of the entire set of genes involved in the assembly of TCP may be one of the first steps in colonization of the human intestine.

Notably, despite high levels of *tcpA* expression in early human infection, we did not observe high levels of expression of genes encoded by CTXϕ, including *ctxAB*, in either early or late human infection. This is consistent with other published results (3) and points to important differences between the regulation of virulence gene expression in the human intestine and that in in vitro models, where *tcpA* and *ctxAB* are coordinately expressed. Indeed, during the course of human infection we additionally observed an uncoupling in expression of the two upstream regulators of *tcpA* and *ctxAB*. In particular, *toxR* expression did not differ between the two phases of human infection (fold change, 1.08 [$P = 0.57$]), nor did that of its accessory transmembrane protein *toxS* (fold change, 0.97 [$P = 0.61$]). In contrast, *tcpP* (fold change, 4.17 [$P = 0.06$]) and its accessory transmembrane protein *tcpH* (fold change, 12.02 [$P = 0.06$]), both encoded on the TCP island, were each more highly expressed in early than in late human infection, al-

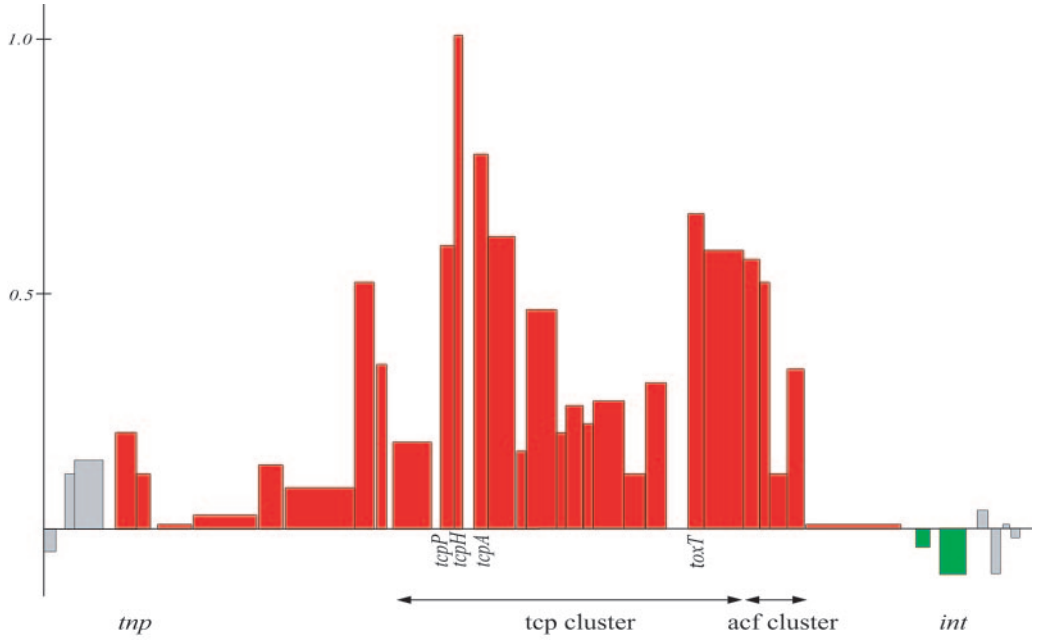

FIG. 2. Differential expression of genes in the TCP island during early compared with late human infection, represented as $\log_{10}$ fold change. Expression of the transposase sequence (*tnp*) of the TCP island is shown at the left, and that of the integrase gene (*int*) is at the right. Transcripts in the TCP island that are more highly expressed in early human infection are shown in red, and transcripts that are more highly expressed in late human infection are shown in green. Genes flanking the TCP island are represented in grey. The differential expression of the individual gene *tcpA* achieved statistical significance after adjustment with the false discovery rate control.

though the fold changes did not achieve statistical significance (Fig. 1). These findings should be confirmed with additional human samples; they suggest that *tcpPH* may play an earlier role in the activation of *V. cholerae* virulence gene expression in vivo than *toxRS*.

DISCUSSION

Here we have used a microarray-based approach to directly study the gene expression pattern of *V. cholerae* during two phases of human infection. We observed that the expression of a key virulence factor, *tcpA*, is much more prominent in the early than the late phase of human infection. On the other hand, high levels of *ctxAB* expression were not observed in vibrios recovered from either human vomit or stool. This could indicate that natural infection requires only a basal level of expression of *ctxAB*. Alternatively, CTX production may take place in a unique intestinal microenvironment that is not represented by our samples, such as in the more distal small intestine or in a subset of organisms that have attached to the intestinal epithelium. Animal studies support the latter hypothesis. In particular, studies using recombinase-based in vivo expression technology with the infant mouse model of cholera indicate that the production of cholera toxin is spatially separate from and temporally dependent on the prior expression of *tcpA* (18). In our study, *toxRS* and *tcpPH*, the two regulatory complexes that have been shown in vitro to together control the expression of *V. cholerae* virulence genes, also were uncoupled during early human infection. Together, these findings illustrate the complexity of the environmental signals experienced by *V. cholerae* during its passage through the human host and underscore the difficulty of fully capturing these dynamic interactions with laboratory-based models.

Our results also have implications for the development of improved therapeutics and vaccine strategies for cholera. The *V. cholerae* colonization factor TCP is very highly expressed during the earliest stage of human infection, along with a number of novel virulence genes. Studies with North American volunteers and with cholera patients from Indonesia had previously suggested that TCP was not strongly immunogenic during natural cholera infection (8). However, recent work in Bangladesh using recombinant *V. cholerae* O1 El Tor TcpA has shown that cholera patients in fact mount substantial mucosal and systemic immune responses to the major subunit of TCP (1). Overall, 93% of patients studied showed a TcpA-specific mucosal or systemic response. High-level expression of *tcpA* in the human upper intestine, combined with its potent immunogenicity, suggests that research on the role of immunity to TcpA in protection from cholera is warranted. Further studies of the two putative hemolysins and the hypothetical proteins identified in our analysis may also identify novel therapeutic targets.

Our evaluation of the gene expression pattern of *V. cholerae* observed directly in clinical specimens also highlights an important evolutionary relationship between this microbe and the human host. *V. cholerae* is unique among the major diarrheal pathogens because it is part of the free-living bacterial flora of aquatic environments. Through a series of incompletely understood events, strains of *V. cholerae* emerge from estuarine waters to cause widespread human disease. Our findings indicate that the human upper intestine is a particularly suitable niche for replication of *V. cholerae* outside the aquatic envi-

ronment. This may in itself represent an evolutionary strategy for dissemination, since the organism is shed in prodigious quantities from an infected person ($>10^8$ CFU/milliliter of stool), and such organisms appear to exist in a hyperinfectious state for the next host (19). Additionally, the human upper intestine may be a particularly well suited environment for the acquisition of foreign genetic material that is important in the evolution of pathogenic *V. cholerae* strains. Studies with the suckling mouse model of cholera have demonstrated the transfer of CTXϕ between bacterial strains in vivo (17, 24). Our transcriptional data suggest that optimal conditions for CTXϕ transduction of *V. cholerae* exist during early infection of the human host, the only known reservoir for the organism outside of estuarine environments. Infection in the human intestine may thus foster the development of pathogenic *V. cholerae* strains, as well as enriching for their multiplication and subsequent dissemination.

In this study, we have taken advantage of the large quantities of vibrios present in clinical samples in order to study an important human pathogen within the host environment. With the refinement of genome-based techniques, similar studies of other microbial pathogens within specific human environments will become increasingly feasible and may lead to new insights into bacterial virulence.

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